Embryonic stem cells can form germ cells *in vitro*

Yayoi Toyooka, Naoki Tsunekawa, Ryuko Akasu, and Toshiaki Noce*

Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya Machida-shi, Tokyo 194-8511, Japan

Edited by David L. Garbers, University of Texas Southwestern Medical Center, Dallas, TX, and approved July 17, 2003 (received for review May 14, 2003)

Knock-in embryonic stem (ES) cells, in which *GFP* or *lacZ* was expressed from the endogenous mouse vasa homolog (*Mvh*), which is specifically expressed in differentiating germ cells, were used to visualize germ cell production during *in vitro* differentiation. The appearance of MVH-positive germ cells depended on embryoid body formation and was greatly enhanced by the inductive effects of bone morphogenic protein 4-producing cells. The ES-derived MVH-positive cells could participate in spermatogenesis when transplanted into reconstituted testicular tubules, demonstrating that ES cells can produce functional germ cells *in vitro*. *In vitro* germ cell differentiation provides a paradigm for studying the molecular basis of germ line establishment, as well as for developing new approaches to reproductive engineering.

Germ-line cells are responsible for transmitting genetic information and for reproducing totipotency from generation to generation. Pluripotent stem cell lines, embryonic stem (ES) cells, and embryonic germ cells are established from cells of the germ cell lineage. Therefore, germ cell specification must be linked to the maintenance of pluripotency, as well as to cell fate commitment leading to gametogenesis.

Unlike many animal species, in which the germ line is predetermined by maternal factors, germ cell specification in mammals takes place at the onset of gastrulation, after implantation of the embryo. In the mouse, primordial germ cells (PGCs) are first distinguished at the base of the allantois in gastrulating embryos at embryonic day (E) 7.25 (1). Lineage studies of epiblast cells show that mouse PGCs are specified by inductive interactions at the onset of gastrulation (2, 3). Genetic analyses using targeted mutations have revealed that bone morphogenic protein (BMP) 4 and -8b, soluble growth factors belonging to the transforming growth factor β superfamily that are produced by extraembryonic ectoderm close to the boundary with the proximal epiblast, are required for the generation of PGCs from epiblast cells (4, 5). Moreover, primary cultures of epiblast fragments from embryos at E5.5–E6.0 generate migrating PGCs when they are cocultured with extraembryonic ectoderm (6), and culturing of whole epiblasts from E6.0 embryos on feeder cells expressing both BMP4 and BMP8b gives rise to PGCs (7). These results reveal that BMPs derived from the extraembryonic ectoderm play crucial roles in PGC determination in the proximal epiblast.

Despite such developments, it is not yet known how the founder population of PGCs is segregated from other pluripotent epiblast cells that form somatic cells. To approach this question, we examined the production of germ cells by an established pluripotent ES cell line. ES cells can form all cell lineages when introduced into host blastocysts and give rise to various somatic cell lineages in culture. However, it is not known whether they can generate the germ cell lineage in culture in the absence of the morphogenetic events associated with gastrulation. It has been difficult to address this question without a molecular marker that distinguishes differentiating PGCs from undifferentiated ES cells. Alkaline phosphatase staining and OCT4 expression, which are usually used to identify murine PGCs, cannot be used because ES cells are also positive for these markers (8). Our previous studies have shown that expression of a mouse vasa homolog (*Mvh*) encoding an ATP-dependent RNA helicase is specific for differentiating germ cells from the late migration stage to the postmeiotic stage (9–11). Loss of *Mvh* function causes a deficiency in the proliferation and differentiation of male germ cells (12). We have therefore used a knock-in at the *Mvh* locus to detect the emergence of PGCs from ES cells *in vitro*. The results demonstrate the generation of PGCs from ES cells in culture and show that these ES-derived germ cells have the capacity to form sperm.

Materials and Methods

Generation of Recombinant ES Cell Lines and Culture. *Mvh* DNA clones were isolated from a mouse (129/Svj) FixII genomic library (Stratagene), and the genomic organization of the *Mvh* locus from exon 1 to exon 11 was determined (Fig. 1). A 2.2-kb *Xba*I–*Spe*I 5′ fragment and a 1.2-kb *Xba*I–*Xho*I 3′ fragment were used as homologous regions to construct targeting vectors. A 3.0-kb fragment containing the first ATG (exon 2) was replaced by an *IRES-lacZ* or *IRES-GFP* cassette and a *Pgk-Neo* cassette, and a *Tk-DTA* cassette was attached to the 3′ end for negative selection. After electroporation of the vectors into 2×10^7 E14TG2a ES (XY) cells, the desired recombination events were verified by Southern blotting, by using as probes a 0.7-kb *Hin*dII–*Xba*I and a 2.6-kb *Eco*RI–*Pst*I fragment external to the 5′ and 3′ ends of the targeting construct. Two *lacZ* knock-in ES clones (nos. 417 and 235) and one *GFP* knock-in ES clone (no. 37) were used in this study. Suspension cultures permitting cell aggregation and embryoid body (EB) formation were set up in suspension culture-grade dishes. ES cells were suspended in DMEM containing 10% FCS at $\approx 1–2 \times 10^6$ cells per milliliter, and, in the case of mixed aggregation cultures, equal numbers of effector cells such as M15-derived cells were mixed with the ES cells. M15 cell lines producing BMP4 were generated by transformation with *BMP4-IRES-Neo* driven by cytomegalovirus promoter. Trophoblast-derived cells were prepared as described (13).

RT-PCR. PolyA RNA from EB cultures was extracted with a Micro-FastTrack 2.0 kit (Invitrogen). RT-PCR was performed with SuperScript One-Step RT-PCR with platinum *Taq* (Invitrogen) according to the manufacturer's instructions. The specific primers were 5′-CTCGAACCACATCCTTCTCT-3′ and 5′-GGCGTTCTCTTTGGAAAGGTGTTC-3′ for the amplification of *Oct4*, 5′-GCTCAAACAGGGTCTGGGAAG-3′ and 5′-GGTTGATCAGTTCTCGAG-3′ for *Mvh*, 5′-TGCCTTTGCTCCGCACCAT-3′ and 5′-GGGTGAAGCACTTCAGGACC-3′ for *Fragilis*, 5′-GCAATCTTGTTCCGAGCTAG-3′ and 5′-CTGGATCGTTGTGCATCCTA-3′ for *Stella*, and 5′-GAGATGGCTGCGCGTCCGGGA-3′ and 5′-CTCAGTGGCAGCCACAGGCCT-3′ for *BMP8b*. *G3PDH* primers used as a control were supplied with the RT-PCR kit. The cycling conditions were as follows: 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C (25 cycles).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PGC, primordial germ cell; EB, embryoid body; ES, embryonic stem; BMP, bone morphogenic protein; GCNA1, germ cell nuclear antigen 1; MVH, mouse vasa homolog; E*n*, embryonic day *n*; SYCP3, synaptonemal complex protein 3.

*To whom correspondence should be addressed. E-mail: noce@libra.ls.m-kagaku.co.jp.

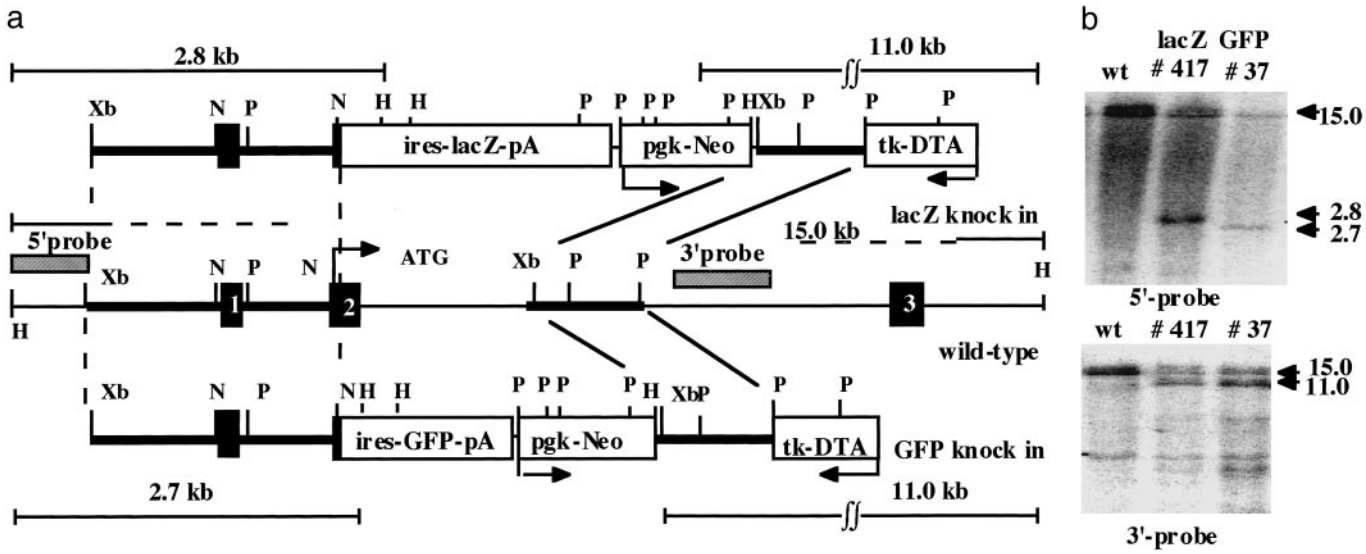

Fig. 1. Targeted replacement of the *Mvh* gene. (*a*) Genomic organization of the *Mvh* locus and structure of the targeting vectors used to replace the 3.0-kb region containing the translation initiation site with *IRES-lacZ* or *IRES-GFP* and *pGK-Neo*. Probes used for Southern blots are indicated by shaded bars. (*b*) Southern blots of *Hin*dIII-digested genomic DNA from the ES clones. The wild-type, *lacZ* knock-in, and *GFP* knock-in loci gave, respectively, 15.0-, 2.8-, and 2.7-kb bands with the 5′ probe, and 15.0-, 11.1-, and 11.0-kb bands with the 3′ probe. B, *Bam*HI; H, *Hin*dIII; N, *Not*I; P, *Pst*I; Xb, *Xba*I.

Cell Preparation and Immunostaining. EBs were dissociated by incubation for 30 min in PBS containing 0.01% type-I collagenase (GIBCO), and the cells were stained for β-gal by using ImageneGreen C_{12} FDG lacZ Gene Expression Kit (Molecular Probes). They were analyzed and sorted with a FACStar PLUS (Becton Dickinson). Dead cells stained by propidium iodide (PI) were excluded from the analysis. For immunocytochemical staining, ES-derived cells and testicular tubules were fixed in 4% paraformaldehyde in PBS and stained with anti-MVH, germ cell nuclear antigen 1 (GCNA1), synaptonemal complex protein 3 (SYCP3), β-gal (Cappel), or HSC70t antibodies in conjunction with horseradish peroxidase-, Texas red-, or FITC-conjugated secondary antibodies (Molecular Probes), as described (10, 14).

Transplantation of Cell Aggregates to the Testis Capsule. Male gonadal cells were prepared from E13.5 embryos of four pregnant mice (Slc:ICR). *LacZ*-positive cells were purified twice by fluorescence-activated cell sorting (FACS) of 1-day coaggregate cultures of *lacZ* knock-in ES and BMP4-producing M15 cells. Approximately 4×10^5 gonadal cells and $\approx 1 \times 10^5$ FACS-purified *lacZ*-positive cells (>95% in purity) were mixed for 16 h in 96-well plates (Sumilon, Tokyo), and the resulting tight aggregates were transplanted under the testis capsules of male CD-1 (ICR) nude mice (Charles River Breeding Laboratories). Sperms were collected from the lumen of sections of transplant or wild-type seminiferous tubules, and DNA was extracted after filtration and centrifugation to remove cellular contamination. All procedures involving mice were carried out in accordance with institution guidelines and with institution permission.

Results

Detection of MVH-Positive Cells During EB Formation by Using *Mvh* Knock-In ES Cells. The targeting schema used to generate *Mvh* knock-in ES cell lines carrying the *GFP* or *lacZ* genes (*Mvh-GFP* and *Mvh-lacZ* lines) is shown in Fig. 1. Screening for the desired homologous recombination event resulted in the isolation of four different knock-in ES cell lines following transformation with *GFP* (no. 37) and *lacZ* (nos. 146, 235, and 417) knock-in vectors. In a routine culture on tissue-culture dishes in a medium containing lymphocyte inhibitory factor (LIF), the knock-in cells did not express the reporter gene products. However, when they were cultured as aggregates in a LIF-free medium and formed EBs, approximately 1/10th of the EBs contained *GFP*- or *lacZ*-positive cells. These appeared around day 3, and their number reached a plateau by day 5. They appeared at first to be randomly distributed in the aggregates (Fig. 2 *a–h*), but clustered together at the periphery of the EBs after days 5–7 as EB formation progressed (Fig. 2*i*). No *GFP*- or *lacZ*-positive cells were observed when the ES cells differentiated as monolayers in LIF-free medium (data not shown).

The *GFP*- or *lacZ*-positive cells were purified by flow cytometry from dissociated 5-day-old EBs, and their germ cell characteristics were further examined. The positive cells were stained with anti-MVH antibody, whereas the negative cells and undifferentiated ES cells were not, thus showing that the positive cells produced endogenous MVH protein as well as the reporter proteins (Fig. 2 *j* and *l*). They also stained for Oct3/4, E-cadherin, SSEA1, c-KIT, and alkaline phosphatase (data not shown), although these properties are shared by pluripotent embryonic cells and PGCs before and after colonizing the fetal gonad. The MVH-positive cells purified from EBs also stained for GCNA1 and SYCP3 (Fig. 2 *j–m*), which are specific for PGCs after they colonize the fetal gonad (10, 15–17), and are not expressed by ES cells. Taken together, these results indicate that the MVH-positive cells are equivalent to germ cells that have colonized the fetal gonad *in vivo*.

BMP4-Producing Cells Enhance PGC Differentiation from ES Cells. Previous studies on gene-targeted mice have revealed that BMP4 and BMP8b, which are produced by the extraembryonic ectoderm (trophoblast precursor), play essential roles in the formation of germ cells in the proximal region of the epiblast (4, 5). Accordingly, to investigate whether the inducing effect of BMPs can be reproduced in culture, the knock-in ES cells were cocultured with 7.5-day-old trophoblast cells, or M15 cells (18) expressing either BMP4 or BMP8b. Notably, MVH was expressed within a day when ES cells were coaggregated with trophoblast- or BMP4-producing M15 cells (Figs. 3 and 4 *a–f*). The effect of BMP4 was not restricted to M15 cells: a similar effect was obtained with other BMP4-producing cell

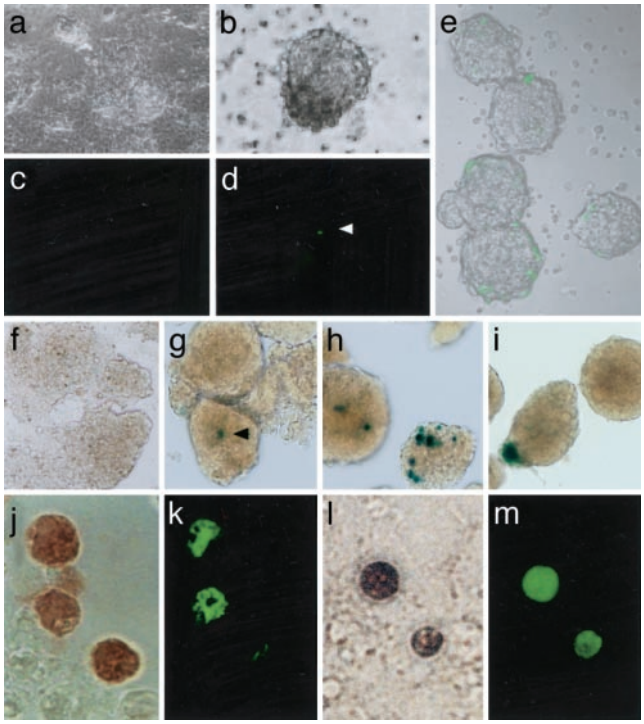

Fig. 2. *In vitro* differentiation of knock-in ES cell EBs. Phase contrast (*a* and *b*) and fluorescence (*c* and *d*) images of GFP knock-in ES cells cultured in the undifferentiated state (*a* and *c*), and of EBs after 3 days of culture (*b* and *d*; arrow indicates a GFP-positive cell). Shown is a confocal microscopic view merging phase contrast and fluorescence images of EBs after 5 days of culture (*e*). 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining of *lacZ*-knock-in ES cells; undifferentiated state (*f*), 3-day EBs (*g*; arrow indicates blue-stained cells expressing *lacZ*), 5-day EBs (*h*), and 7-day EBs (*i*). *LacZ*-positive cells were partially purified from the 5-day EBs and stained with anti-MVH to detect endogenous MVH expression (*j* and *l*). Partially purified cells were double-stained with anti-MVH (*j*) and GCNA1 (*k*) antibodies, or anti-MVH (*l*) and anti-SYCP3 (*m*) antibodies. Anti-MVH staining was detected with horseradish peroxidase-conjugated secondary antibody, and GCNA1 and anti-SYCP3 staining was visualized with FITC-conjugated secondary antibody.

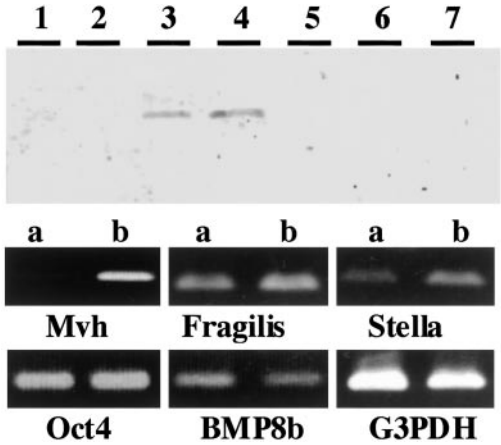

Fig. 3. MVH expression and RT-PCR analyses of coaggregation cultures of knock-in ES and effector cells. (*Upper*) Immunoblot with anti-MVH. Extracts (20 μg of protein) of 1-day aggregation culture of ES cells (lane 1), ES cells coaggregated with wild-type M15 cells (lane 2), ES cells coaggregated with BMP4-producing M15 cells (lane 3), ES cells coaggregated with trophoblast cells (lane 4), ES cells coaggregated with STO cells (lane 5), BMP4-producing M15 cells (lane 6), and trophoblast cells (lane 7). (*Lower*) Poly(A) RNA from undifferentiated ES cells (lanes a) and from coaggregates of ES and BMP-producing cells after 1 day (lanes b) was used for amplification by using primer sets specific for each of the genes indicated below the panels.

lines, such as 3T3 (NIH) or STO cells carrying a BMP4 expression vector. On the other hand, BMP8b had little effect on the emergence of PGCs (data not shown). MVH-positive PGCs appeared after 1 day in almost all of the cell aggregates in mixtures with BMP4-producing cells (Fig. 4*b*), suggesting that the number of PGCs increased in culture, and this was confirmed by quantitative fluorescence-activated cell sorter analysis. Almost no MVH reporter-positive cells (<0.01%) were found in 1-day-old aggregates of ES cells on their own or in coaggregates with wild-type M15 cells (Fig. 4 *c*, *d*, and *g*). Only 0.4 ± 0.2% of the cells were positive in 5- to 7-day-old EBs derived from ES cells on their own, whereas they constituted 2.9 ± 0.7% of the cells in 1-day coaggregates with BMP4-producing M15 cells (Fig. 4*h*). By sorting the positive cells twice we obtained >95% pure MVH-positive PGCs (Fig. 4*i*). In addition, when we inhibited binding of BMP4 to the ES cells by adding a fusion protein consisting of IgG and ALK3 (BMP receptor protein) (19), the yield of PGCs was markedly reduced (Fig. 4*j*). These data suggest that BMP4 exposure in cell mass play a crucial role for determining the time of emergence and frequency of PGCs from ES cells in culture.

ES-Derived PGCs Can Differentiate into Sperm. We carried out transplantation experiments to examine the developmental potential of the ES-derived MVH-positive cells. Gonadal cells dissociated from E12.5–E15.5 gonads can reorganize into gonadal tissues when aggregated in suspension culture and transplanted into adult gonads (20, 21). Accordingly, after 1 day of coculture of male *Mvh-lacZ* knock-in ES cells with BMP4-producing M15 cells, *lacZ*-positive ES-derived cells were purified by cell sorting and cocultured with gonadal cells dissociated from E12.5–E13.5 male gonads. The combined aggregates were transplanted under a host testis capsule. Six to 8 weeks after transplantation, the transplants had formed testicular tubules separated from the host tubules (in four of six independent experiments), and ES-derived *lacZ*-positive cells were detected inside these tubules. Histological analysis showed that they were in the inner cell layers of the tubules and that there were mature sperm in the lumens (Fig. 5 *a–c*). This finding indicates that ES-derived PGCs can be differentiated into Mvh-expressing spermatogenic cells, because strong Mvh expression in the adult testis is characteristic of meiotic cells from the pachytene spermatocyte to the round spermatid stage (10). As a control, aggregates of male gonadal cells were transplanted in the absence of ES-derived cells; they also formed tubules, but germ cell layers were rarely observed and sperm was never found in the tubules. When undifferentiated ES cells or unsorted ES cells from EBs, instead of purified *lacZ*-positive ES-derived cells, were used for transplantation, they formed teratomas, and there was no β-gal staining in the transplants (Fig. 5 *d* and *e*).

To confirm that the ES-derived *lacZ* cells go through meiosis and spermiogenesis, the transplanted tubules were double-stained with anti-β-gal to detect ES-derived MVH-positive cells and anti-HSC70t to detect elongated spermatids (14). As shown in Fig. 6*A*, all of the germ cells in sections of seminiferous tubules stained with anti-β-gal, indicating that they were derived from *Mvh-lacZ* ES cells, whereas somatic cells such as Sertoli cells stained negative with anti-β-gal (Fig. 6*A g* and *h*). The β-gal-positive cells in the innermost layer had characteristic elongated nuclei and were stained with anti-HSC70t, demonstrating that they were elongated spermatids derived from ES cells (Fig. 6*A*, *d–j*). Finally, PCR analysis of DNA

DEVELOPMENTAL BIOLOGY

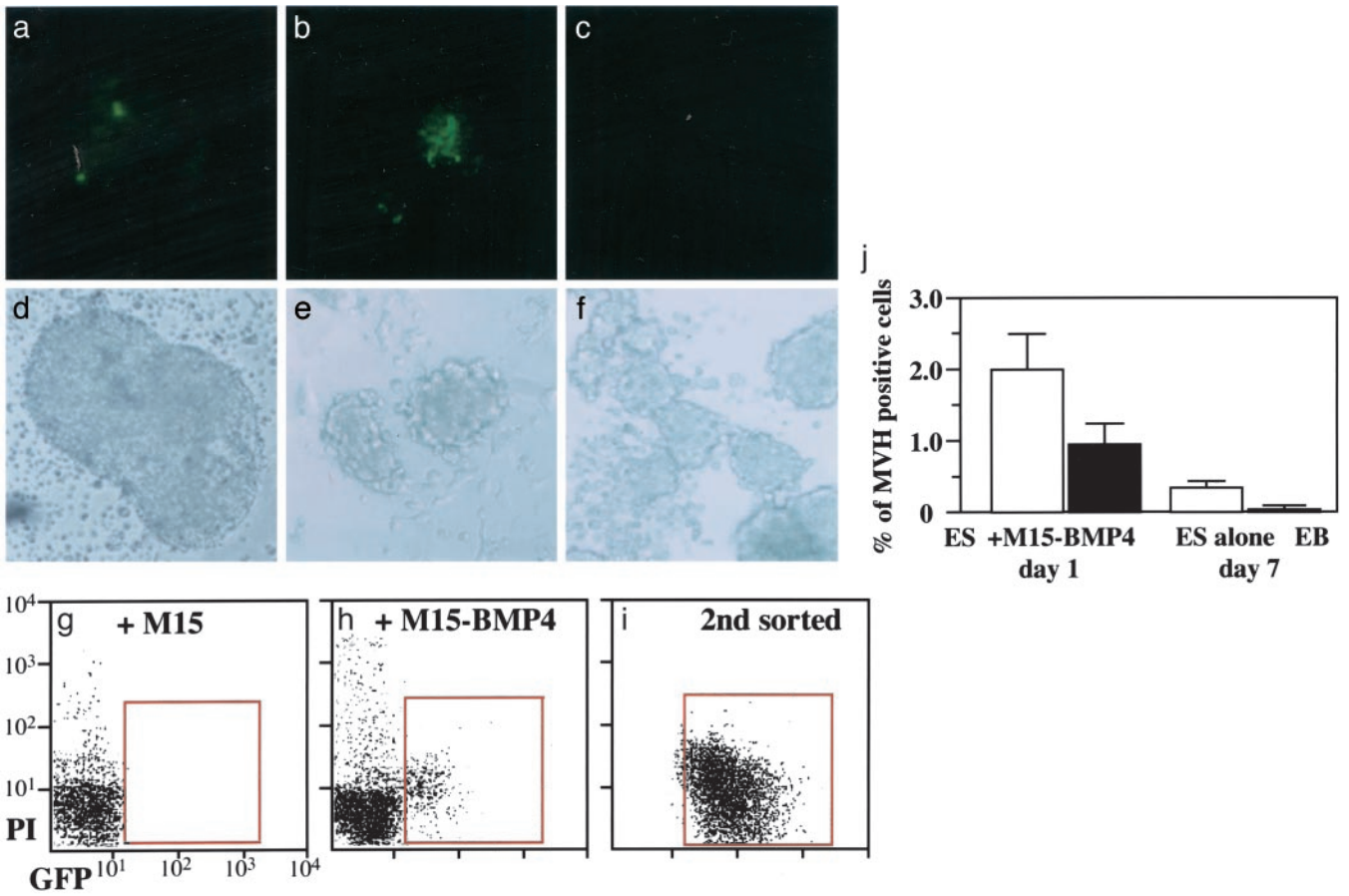

Fig. 4. Coaggregation cultures of knock-in ES and effector cells. *GFP*-knock-in ES cells were cultured for 1 day as aggregates with trophoblast cells prepared from E7.5 embryos (*a* and *d*), BMP4-producing M15 cells (*b* and *e*), and the wild-type M15 cells (*c* and *f*). (*d–f*) Phase contrast views of the upper fluorescence images in *a–c*, respectively. (*g–i*) Flow cytometric analyses of cells from 1-day-old aggregates of GFP knock-in ES cells mixed with wild-type M15 cells (1:1) (*g*), the same cells cultured for 1 day with BMP4-producing M15 cells (*h*), and GFP-positive cells sorted once from the cells in *h* (*i*). (*j*) Inductive effect of BMP4/BMP8b and inhibitory effect of ALK3-IgG fusion protein on germ cell differentiation *in vitro*. Cell-free supernatants of normal Cos7 cells (open bars) or ALK3-IgG-producing Cos7 cells (filled bars), were added at a 2× dilution to cultures of ES cells coaggregated with BMP4-producing M15 cells for 1 day or to EBs formed by ES cells for 7 days. Values are derived from flow cytometric analyses of four independent experiments.

extracted from sperm in the transplanted seminiferous tubules revealed the presence of sperm derived from *Mvh* knock-in ES cells (Fig. 6*B*).

Discussion

The differentiation of ES cells *in vitro* provides an excellent model for studying cell commitment and the potential of stem cell technology (22, 23). We have demonstrated that ES cells are able to differentiate into germ-line cells *in vitro* and that these germ-line cells are at least equivalent to the PGCs that migrate into the fetal gonad, and have the potential to undergo meiosis and produce sperm. In addition, we have shown that coaggregate culture with BMP4-producing cells as effectors permits efficient *in vitro* differentiation of the ES cells into germ-line cells. Previous studies have shown that the addition of BMP4 (100–500 ng/ml) induced the formation of PGCs by cultured epiblasts (E5.5–E6.5), but that the PGCs became alkaline phosphatase-positive only at the migratory stage (24, 25). In contrast, we observed an inductive effect of BMP4 only when the ES cells were coaggregated with the BMP4-producing cells; no induction was found when BMP4 was added to a suspension culture of ES cells or when ES cells were cultured in tissue culture dishes with BMP4-producing cells as feeder cells (data not shown). This appears to agree with the lack of an additive effect of BMP4-producing feeder cells on primary cultures of epiblast tissue (7). It seems, therefore, that the production of germ cells from ES cells depends on the constant stimulation of BMP4, together with a unique three-dimensional configuration of the EBs. The latter could produce a community effect mimicking close cell association in epiblast tissues.

It is worth noting that only a limited number of ES cells became PGCs in culture. This is consistent with the situation in normal development, in which a limited number of the cells in the proximal epiblast are allowed to differentiate into germ cells. Assuming that the ES cells in the coaggregates are uniformly exposed to the BMP4, it would seem that the "choice" of germ cell fate may depend on differences among the ES cells, and that the choice is then reinforced by interactions between the cells becoming germ cells and those retaining the somatic cell fate. Recent studies have revealed that the intracellular signaling proteins, SMAD1 and SMAD5, acting downstream of BMP receptors, are critical for the derivation of the germ cell linage from the epiblast (25–27). Germ cell differentiation from clonally established cells should enable us to examine the effect of different culture conditions on germ cell formation, and to use gene transfer techniques such as RNAi and the inducible expression of signaling genes to identify factors involved in germ cell spec-

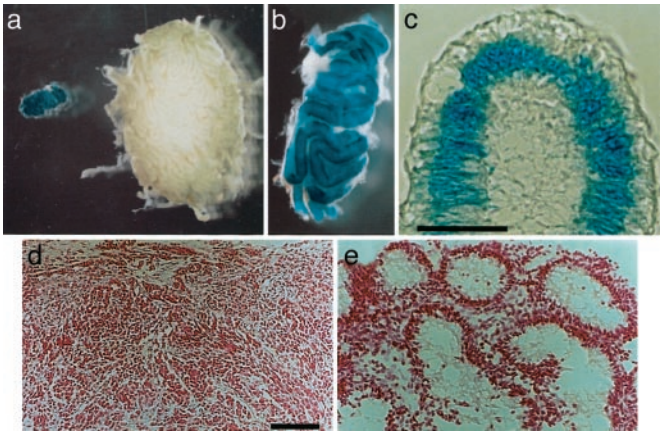

Fig. 5. Transplantation of ES-derived germ cells into adult testis. (*a*) At 5–6 weeks after transplantation, host testes and transplants were stained with 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). (*b*) A magnified view of the transplant. (*c*) A representative view of seminiferous tubules in a section of the transplant. (*d*) A section of the transplant involving unpurified ES cells and embryonic gonadal cells. (e) A transplant of gonadal cells on their own. (Scale bars = 100 μm.)

ification. Moreover, the ES cell system permits the examination of a large number of PGC-competent cells, which would be essential for comprehensive studies such as proteome analysis.

It has been reported that genes involved in the differentiation of the three germ layers, and genes specific for several cell lineages, are expressed in EBs over the same time period as in gastrulating embryos (28). In contrast, the most remarkable feature of the ES-derived germ cells is that germ cell differentiation takes place much faster *in vitro* than *in vivo*. We observed the first appearance of MVH-positive cells from ES cells within 1 day of coculture with BMP4-producing cells, whereas MVH-expressing PGCs first appear in embryos around E9.5, almost 3 days after germ cell determination. One reason for this discrepancy may be that some ES cells in culture have already acquired the capacity to form PGCs. Thus, as shown in Fig. 3, the mRNAs of the PGC founder-specific genes *Stella* and *Fragilis* (29) are detectable in ES cells before the onset of differentiation, whereas those genes are not expressed in the inner cell mass and early epiblast cells *in vivo*. Another possibility is that progression of PGC differentiation may be suppressed in the embryonic environment until the PGCs reach an appropriate position near the developing gonads. It may well be that some of the factors that regulate the timing of PGC differentiation in the embryo are absent from our culture systems. Assuming that the *in vitro* culture system reproduces essential aspects of normal development, the rapid differentiation of PGCs *in vitro* may provide a clue to vital aspects of germ cell development.

Recently, differentiation of mature oocytes from ES cells has been reported (30), demonstrating the derivation of meiosis-competent cells from ES cells in culture, consistent with the results of this study. Thus, *in vitro* culture systems may open the way to an analysis of the gene functions involved in segregation of the germ cell and somatic cell lineages, as well as to a novel approach to reproductive engineering.

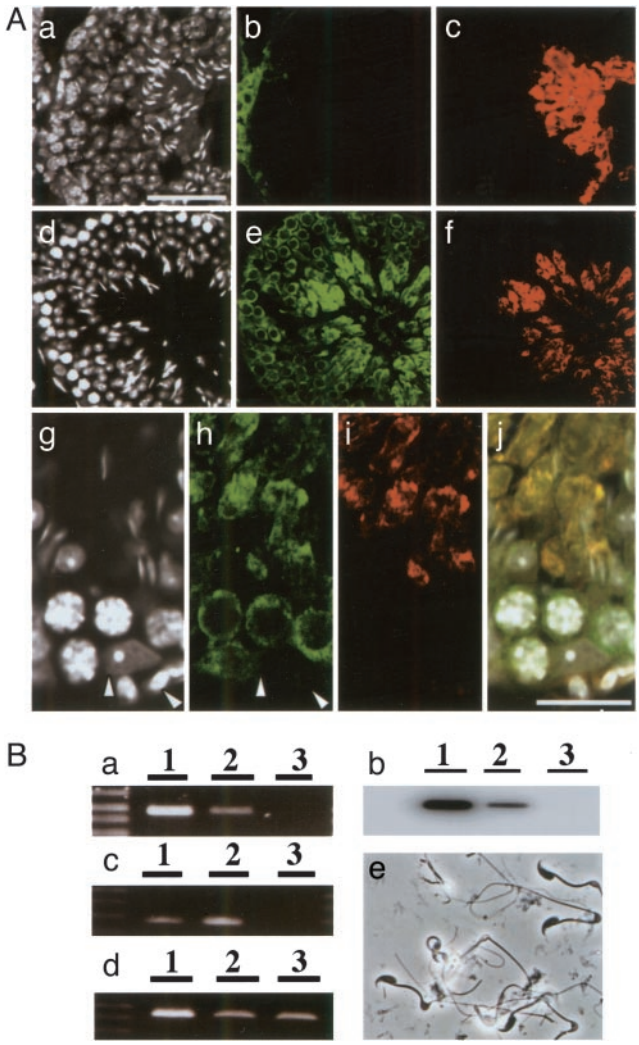

Fig. 6. (*A*) Sections of wild-type (*a–c*) testicular tubules and transplant (*d–j*) tubules double-stained with anti-β-gal and affinity-purified anti-HSC70t antibodies. Shown is nuclear staining (*a*, *d*, and *g*), anti-β-gal staining (*b*, *e*, and *h*), and anti-HSC70t staining (*c*, *f*, and *i*). (*j*) Higher magnification (×6) of a stained transplant tubule and merged image of *g–i*. Arrowheads in *g* and *h* indicate positions of Sertoli and myoid cell. (Bar in *a* for *a–f* is 50 μm and in *j* for *g–j* is 20 μm.) (*B*) Detection of the knock-in allele in genomic DNA extracted from *Mvh-lacZ* knock-in ES cells (lane 1), sperm from a *lacZ*-positive transplant (lane 2), and a wild-type seminiferous tubule (lane 3). Primer pairs detecting *lacZ* (*a*), *Neo* (*c*), and *Sry* (*d*) genes were used for PCR (30 cycles; 94°C, 58°C, and 72°C at 1 min each). (*b*) Southern blot of the PCR product hybridized with labeled *lacZ* probe. (e) Photograph of sperm purified from transplant seminiferous tubules.

We thank Drs. S. Nishikawa and M. Ogawa for technical assistance and helpful collaboration, and Drs. J. May II, H. and N. Nakatsuji, and S. Chuma for kindly providing GCNA1 and SYCP3 antibodies. This work was supported in part by a grant-in-aid from the Ministry of Education, Science, and Culture, Japan.

1. Ginsburg, M., Snow, M. H. L. & McLaren, A. (1990) *Development (Cambridge, U.K.)* **110,** 521–528.
2. Lawson, K. A. & Hage, W. J. (1994) *Ciba Found. Symp.* **182,** 68–91.
3. Tam, P. P. & Zhou, S. X. (1996) *Dev. Biol.* **178,** 124–132.
4. Lawson, K. A., Dunn, N. R., Roelen, B. A., Zeinstra, L. M., Davis, A. M., Wright, C. V., Korving, J. P. & Hogan, B. L. (1999) *Genes Dev.* **13,** 424–436.
5. Ying, Y., Liu, X. M., Marble, A., Lawson, K. A. & Zhao, G. Q. (2000) *Mol. Endocrinol.* **14,** 1053–1063.
6. Yoshimizu, T., Obinata, M. & Matsui, Y. (2001) *Development (Cambridge, U.K.)* **128,** 480–490.
7. Ying, Y., Qi, X. & Zhao, G. Q. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 7858–7862.
8. Yeom, Y. I., Fuhrmann, G., Ovitt, C. E., Brehm, A., Ohbo, K., Gross, M., Hubner, K. & Scholer, H. R. (1996) *Development (Cambridge, U.K.)* **122,** 881–894.

9. Fujiwara, Y., Komiya, T., Kawabata, H., Satoh, M., Fujimoto, H., Furusawa, M. & Noce, T. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12258–12262.
10. Toyooka, Y., Tsunekawa, N., Takahashi, Y., Matsui, Y., Satoh, M. & Noce, T. (2000) *Mech. Dev.* **93,** 139–149.
11. Noce, T., Okamoto, I. S. & Tsunekawa, N. (2001) *Cell Struct. Funct.* **26,** 131–136.
12. Tanaka, S. S., Toyooka, Y., Akasu, R., Katoh-Fukui, Y., Nakahara, Y., Suzuki, R., Yokoyama, M. & Noce, T. (2000) *Genes Dev.* **14,** 841–853.
13. Tanaka, S., Kunath, T., Handjantonakis, A. K., Nagy, A. & Rossant, J. (1998) *Science* **282,** 2072–2075.
14. Tsunekawa, N., Matsumoto, M., Tone, S., Nishida, T. & Fujimoto, H. (1999) *Mol. Reprod. Dev.* **52,** 383–391.
15. Enders, G. C. & May, J. J., II (1994) *Dev. Biol.* **163,** 331–340.
16. Di Carlo, A., Travia, G. & De Felici, M. (2000) *Int. J. Dev. Biol.* **44,** 241–244.
17. Chuma, S. & Nakatsuji, N. (2001) *Dev. Biol.* **229,** 468–479.
18. Larsson, S. H., Charlieu, J.-P., Miyagawa, K., Engelkamp, D., Rassoulzadegan, M., Ross, A., Cuzin, F., Heyningen, V. & Hastie, N. D. (1995) *Cell* **81,** 391–401.
19. Suzuki, A., Shioda, N., Maeda, T., Tada, M. & Ueno, N. (1994) *Biochem. Biophys. Res. Commun.* **198,** 1063–1069.
20. Ohno, S., Nagai, Y. & Ciccarese, S. (1978) *Cytogenet. Cell Genet.* **20,** 351–364.
21. Zenzes, M. T., Wolf, U., Gunther, E. & Engel, W. (1978) *Cytogenet. Cell Genet.* **20,** 365–372.
22. Nishikawa, S.-I., Nishikawa, S., Hirashima, M., Matsuyoshi, N. & Kodama, H. (1998) *Development (Cambridge, U.K.)* **125,** 1747–1757.
23. Rathjen, P. D., Lake, J., Whyatt, L. M., Bettess, M. D. & Rathjen, J. (1998) *Reprod. Fertil. Dev.* **10,** 31–47.
24. Pesce, M., Klinger, G. F. & De Felici, M. (2002) *Mech. Dev.* **112,** 15–24.
25. Hayashi, K., Kobayashi, T., Umino, T., Goitsuka, R., Matsui, Y. & Kitamura, D. (2002) *Mech. Dev.* **118,** 99–109.
26. Tremblay, K. D., Dunn, N. R. & Robertson, E. J. (2001) *Development (Cambridge, U.K.)* **128,** 3609–3621.
27. Chang, H. & Matzuk, M. M. (2001) *Mech. Dev.* **104,** 61–67.
28. Leahy, A., Xiong, J. W., Kuhnert, F. & Stuhlmann, H. (1999) *J. Exp. Zool.* **284,** 67–81.
29. Saitou, M., Barton, C. S. & Surani, A. (2002) *Nature* **418,** 293–300.
30. Hubner, K., Fuhrmann, G., Christenson, L. K., Kehler, J., Reinbold, R., De la Fuente, R., Wood, J., Strauss, J., Boiani, M. & Scholer, H. R. (2003) *Science* **300,** 1251–1256.